Assessment of factors affecting *in vitro* shoot regeneration from axillary bud explant of *Camptotheca acuminata*

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Abstract: Axillary buds from 3-yr.-old seedlings of *Camptotheca acuminata* in the greenhouse were cultured on the different basal media with different concentrations of growth regulators for shoot regeneration for studying the effects of different basal media, different concentrations of growth regulators (BA or TDZ), sucrose, agar and pH value on shoot regeneration from axillary bud. The results showed that B5 and WPM media were the optimal basal media and the optimal phyotohormone was BA of 1.0 mg/L or TDZ of 0.1mg/L; The concentrations of sucrose of 30g/L and agar of 6g/L were most suitable for the shoot regeneration; pH value from 5.8 to 6.6 were broadly effective, but the best at pH 5.8.

Keywords: *Camptotheca acuminata*; Axillary bud; Shoot regeneration; Growth regulators; Basal media

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Introduction

Camptotheca acuminata is a native tree species in China and can produce camptothecin (CPT) that is an anticancer indole alkaloid identified first by Wall *et al.* (1966). CPT and its natural and synthetic analogs have the activity against cancers, including leukemias and cancers of the liver, stomach, breast and colon (Zhang *et al.* 1998). *C. acuminata* has been used as medicinal raw materials because of its increasing economic value. But *C. acuminata* is an endangered species and the extraction of CPT will consume a lot of resource. Moreover, the content of CPT in raw material is very low (Zu *et al.* 2003). In a provenance study, *C. acuminata* seedlings from different seed sources in China and USA had significant differences in CPT contents (Liu *et al.* 1998). So it is highly desirable to develop clonal lines with high CPT-synthesis capabilities for CPT extraction.

In vitro micropropagation offers a low cost, highly efficient technique for propagating medicinal and endangered plants. It is well known that there are many factors affecting plant regeneration, such as basal medium, external phytohormones, sucrose concentration in medium, etc... To our knowledge, there are few reports on detail study for plant regeneration from axillary bud of *C. acuminata*. The objective of this study is to explore the optimal conditions required for induction of adventitious shoots from axillary buds of *C. acuminata*.

Materials and methods

Plant materials

Three–year old seedlings of *C. acuminata* in a greenhouse of Northeast Forestry University were used as sources of axillary buds. Harvested axillary buds were sterilized with 70% ethanol for 30s, 5% (v/v) $NaClO_3$ for 5 min, and subsequently washed three times with sterile water.

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Effect of BA (N^6-benzyladenine) and TDZ (1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea)

Different concentrations of BA (0.5–2.0 mg/L) and TDZ (0.1–0.5 mg/ L) were used to test the effect of growth regulators on shoot induction. WPM medium were used as basal medium with sucrose of 30g/L, and agar of 6g/L at pH 5.8.

Sucrose concentration

The sucrose concentration was 10 g/L, 20 g/L, 30 g/L and 40g/L on shoot induction medium with BA of 1.0 mg/L and agar of 6 g/L at pH 5.8.

Agar concentration

Agar concentration was from 4g/L to 10 g/L with sucrose concentration of 30 g/L, BA of 1.0 mg/L at pH 5.8.

Different pH values

Different levels of pH (4.0, 5.0, 5.4, 5.8, 6.2, 6.6, 7.0) were used to test the effect of pH value on shoot induction. The explants were cultured on WPM medium supplemented with BA of 1.0 mg/L, sucrose of 30 g/L, and agar of 6g/L.

Different basal medium

Three different basal media, MS, B5 and WPM media supplemented with BA of1.0mg/L, sucrose of 30g/L, and agar of 6g/L, at pH 5.8 were used to test the effect of medium compositions on shoot induction.

Culture condition

In this study, all the above cultures conditions were at (25±2) °C, under a photoperiod (16-hour light/8 hour dark) and light intensity was 1000–1500 lux.

Data analysis

Regeneration frequencies of axillary buds (%.) was calculated by the number of explants producing shoots divided by the total number of explants after four weeks on the different media The number of shoots per explant was recorded separately after two weeks and four weeks on these media. Each treatment consisted of 20 explants and was triplicated.

Results and discussion

Effect of BA and TDZ on shoot regeneration from axillary bud

Phyotohormone had a significant effect on shoot regeneration for *C. acuminata*. On the phytohormone-free medium (control in Table 1), the axillary buds almost produced single shoot which grew faster than the shoots regenerated from the medium containing phytohormone. In the medium containing BA of 1.0 mg/L, regeneration frequencies were 88% with higher shoot number, 3.6 shoots per explant (Fig. 1, Table 1). But shoot regeneration frequencies decreased when further increasing the concentration of BA (Table 1). TDZ, this cytokinin-mimicking compound was more effective than the true cytokinin BA for inducing shoot organogenesis in some woody species (Hammatt *et al.* 1998; De Bondt *et al.* 1996). In our study, low levels of TDZ also produced better shoot regeneration, compared with higher level of TDZ. The best result was obtained from the medium containing TDZ of 0.1 mg/L. However, further increasing in TDZ concentration, regenerated shoots frequencies and number was decreased. When TDZ concentration reached 0.5 mg/L, large amounts of white-reddish callus were formed at the proximal cut ends of the shoots, but such callus regeneration frequencies were very low (Table 1). Based on our research, the results showed that higher levels of BA (1.0 mg/L) or lower levels of TDZ (0.1 mg/L) were effective for shoot regeneration of *C. acuminata*.

Table 1. Effect of BA and TDZ on axillary bud proliferation

Cyto-kinin/mg·L^{-1}	Regeneration frequencies of axillary buds (%)	Number of shoots per explant	
		Two weeks culture	Four weeks culture
0.0	65	1.0	1.0
BA0.5	88	2.4	2.8
BA1.0	85	1.9	3.6
BA1.5	72	1.3	3.0
BA2.0	48	1.8	1.8
TDZ 0.1	91	1.4	3.0
TDZ0.2	92	1.0	2.8
TDZ0.3	100	1.1	2.3
TDZ0.4	100	0.8	1.7
TDZ0.5	56	1.1	1.3

Fig. 1 Regenerated shoots from axillary bud of *C. acuminata* after 5 weeks.

Effect of sucrose concentration on shoot regeneration from axillary bud

In vitro cultured plants and tissues need an exogenous supply of carbohydrates as sources of carbon because such plants are not fully autotrophic. The concentration of sucrose in the induction medium has been found to affect regeneration frequencies. Furthermore, sugar concentration affects embryogenesis in many species (Ferried *et al.* 1995). In the present study, sucrose concentration had an important effect on shoot regeneration for *C. acuminata* (Table 2).The medium without any sucrose did not support axillary bud regeneration. All axillary buds remained greenish-yellow during the whole culture-period. On the medium containing sucrose, regenerated shoots frequencies and number increased with sucrose concentration at 10–30 g/L. Regeneration frequencies was from 33% to 80%, with increasing shoot number from 1.8 to 2.7 shoots per explant (Table 2). When sucrose concentration was further increased up to 40 g/L, regeneration frequencies and number were both inhibited, i.e. regeneration frequencies was 60% with less shoot number, 1.3 shoots per axillary buds. On the other hand, the sucrose of 40 g/L (i. e higher concentrations) induced small amounts of callus at the proximal ends of the shoots. Therefore, based on our research results, we concluded that sucrose of 30 g/L was the optimal concentration for shoot regeneration in *C. acuminata*. This results is similar to that of Buiteveld *et al.* (1993), but it was contrary to the report by Zheng *et al.* (1998), who reported that different concentrations of sucrose had no significant effect on the plant regeneration of onion and shallot. From the above, we can conclude that during the establishment of a new regeneration system, the effect of sucrose concentration on regeneration efficiency should be studied deeply future.

Table 2. Effect of sucrose concentration on axillary bud proliferation

Sucrose /g·L^{-1}	Regeneration frequencies of axillary buds (%)	Number of shoots per explant	
		Two weeks	Four weeks
0	0	0	0
10	33	1	1.8
20	56	1.6	1.7
30	80	1.5	2.7
40	60	1.3	2.0

Effect of agar concentration on shoot regeneration from axillary bud

It is well known that agar as a solidifying agent can have an effect on the growth and development of *in vitro* cultures (Scholten *et al.* 1998). Our study showed that the agar concentration had a significant effect on shoot regeneration (Table 3). In the medium containing agar of 3 g/L, regeneration frequencies of the explants were 60%, with less shoots, 1.8 shoots per explant. Moreover, agar of 3g/L caused a high level of shoot vitrification (picture not shown here, c.f. Fig. 1). The vitrification is also called as hyperhydricity, or water logging. It is a physiological disorder of *in vitro* grown plants resulting in tissue hypertrophies. The main symptom is too much increases of water in the tissues (Hussey 1986). The result in our study may be due to the fact that the explants cultured at low agar concentration levels were able to uptake more water from the medium to show much higher vitrification (Hussey 1986). On the medium containing agar of 6-8g/L, the explants produced better results. On the me-

dium containing 10g/L agar, the explants produced less shoots with slow growth. In this study, agar concentration at 6 g/L was the optimal.

Table 3. Effect of agar concentration on axillary bud proliferation

Agar /g·L^{-1}	Regeneration frequencies of axillary buds (%)	Number of shoots per explant	
		Two weeks	Four weeks
3	60	1.2	1.8
6	88	1.7	3.2
8	84	1.0	3.4
10	65	1.6	1.8

Effect of pH value on shoot regeneration from axillary bud

It has been reported that the pH value of the medium can regulate the uptake of nutrients by the explants (Hussey 1986). Our study showed that pH level of 5.8–6.6 was broadly effective for shoot regeneration for *C. acuminate* (Table 4). The best result of shoot regeneration was found for the medium at pH 5.8, with regeneration frequencies of 90% and a high shoot number (3.2 shoots per explant). On the medium at pH 7.0 or below pH 5.4, regenerated shoot number was low. Moreover, on the medium with pH value below 5.4, regenerated shoots showed serious vitrification. However, various results were also observed in former studies. For example, it is reported that pH value had no consistent effect on the efficiency of shoot regeneration (Lazzeri *et al.* 1987). In chickpea, pH 6.5 was proved to be the optimum for embryo maturation, which was adversely affected by pH above 7.0 and below 4.0 (Barn *et al.* 1993). These indicated that the effect of pH value on plant regeneration depends on plant species.

Table 4. Effect of pH value on axillary bud proliferation

pH value	Regeneration frequencies of axillary buds (%)	Number of shoots per explant	
		Two weeks	Four weeks
5.0	56	1.1	1.1
5.4	56	1.9	2.9
5.8	90	1.8	3.2
6.2	88	1.6	2.9
6.6	85	1.8	2.6
7.0	70	1.5	2.1

Effect of basal medium on shoot regeneration from axillary bud

Different basal medium had different effects. On the MS medium, regeneration frequencies of axillary buds were very low (only 24%), with 1.8 shoots per explant. WPM and B5 media had better effects for shoot regeneration, and relatively higher shoot regeneration frequencies were obtained (Table 5). It can be concluded that WPM and B5 media are effective basal medium for axillary buds regeneration of *C. acuminata*.

In conclusion, we have established a successful tissue culture system for *C. acuminata* by direct shoot regeneration from axillary bud explant. The results show that B5 and WPM media are the optimal basal media and the optimal phyotohormone is BA of 1.0 mg/L or TDZ of 0.1mg/L; The concentrations of sucrose of 30g/L and agar of 6g/L are most suitable for the shoot regeneration; pH value from 5.8 to 6.6 are broadly effective, but the best at pH 5.8. The results of this study provide scientific data for rapid and economical propagation of mass cloning of *C. acuminata*.

Table 5. Effect of basal medium on axillary bud proliferation

Basal medium	Regeneration frequencies of axillary buds (%)	Number of shoots per explant	
		Two weeks	Four weeks
MS	24	1.0	1.8
WPM	76	1.4	2.3
B5	80	1.3	2.6

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